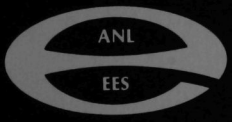
ANL/EES-TM-187

Ethanol Production Via Fungal Decomposition and Fermentation of Biomass

Semiannual Progress Report,
October 1981—March 1982



ARGONNE NATIONAL LABORATORY
Energy and Environmental Systems Division

operated for
U. S. DEPARTMENT OF ENERGY
under Contract W-31-109-Eng-38

MEMBERS OF ARGONNE UNIVERSITIES ASSOCIATION

Printed in the United States of America. Available from National Technical Information Service, U. S. Department of Commerce, 5285 Port Royal Road, Springfield, Virginia 22161.

ANL/EES-TM-187

ARGONNE NATIONAL LABORATORY
9700 South Cass Avenue
Argonne, Illinois 60439

ETHANOL PRODUCTION VIA FUNGAL DECOMPOSITION AND FERMENTATION OF BIOMASS

SEMIANNUAL PROGRESS REPORT, OCTOBER 1981-MARCH 1982

by

Antonios A. Antonopoulos and Edward G. Wene
Energy and Environmental Systems Division

April 1982

Prepared for
Solar Energy Research Institute

Work Sponsored by
U.S. DEPARTMENT OF ENERGY

PREFACE

This semiannual progress report focuses on research efforts and results for the first half of FY 1982 in the Ethanol Production via Fungal Decomposition and Fermentation of Biomass program at Argonne National Laboratory. This work is sponsored and funded by the U.S. Department of Energy (DOE) through the Solar Energy Research Institute (SERI).

CONTENTS

TABLES

FIGURES

ACKNOWLEDGMENTS

The authors extend their appreciation and gratitude to the following individuals: (1) Larry Douglas, Van Morriss, and Clayton Smith of SERI, for their cooperation and support; (2) Ann Gould, mycologist at Argonne, for the zeal and competence she has demonstrated in this study; (3) several investigators and contractors participating in the DOE/SERI Alcohol Fuels Program, for their useful comments; and (4) Charles Malefyt, who edited the manuscript, and Barbara Rogowski, who typed the report.

ETHANOL PRODUCTION VIA FUNGAL DECOMPOSITION AND FERMENTATION OF BIOMASS

SEMIANNUAL PROGRESS REPORT, OCTOBER 1981-MARCH 1982

Antonios A. Antonopoulos and Edward G. Wene

ABSTRACT

During the first half of FY 1982, a few additional Fusarium isolates were obtained and screened for their cellulolytic and fermenting abilities. Experiments with an isolate from Fusarium strain ANL 22-760 showed that cellulose was the best inducer of FPase and CMCase, while glucose, lactose, and xylose almost completely inhibited cellulase production. Tests with Fusarium strain ANL 11182 indicated that a cellulase production of 2.5 IU/mL in 14 days was obtained at 28°C in a medium of 1% cellulose and in the presence of 0.7 mg/mL extracellular protein. Increasing the nitrogen content of the medium stimulated cell growth but decreased cellulase production. The ratio of CMCase to FPase remained nearly constant at about 10:1 during fermentations with varying concentrations of cellulose and nitrogen sources. Several of the new isolates and mutants, when screened for their fermenting ability in glucose, produced ethanol at a conversion efficiency close to that achieved in yeast fermentations. Fermentations of 1% glucose by isolates from strain ANL 99A-78 repeatedly produced 4.3 mg/mL ethanol within 48 hours. A few of the new isolates and mutants produced, in consecutive fermentations of 1% xylose, up to 4.2 mg/mL ethanol within 48 hours. Addition of glucose to higher concentrations of xylose increased the yield of ethanol.

1 EXECUTIVE SUMMARY

Many microorganisms are able to hydrolyze cellulose and hemicellulose, and many others can ferment simple sugars to ethanol. Relatively few, however, are capable of performing all the steps necessary to convert biomass to ethanol. Recorded evidence indicates that selected Fusarium strains are able to partially free celluloses from their lignin mantle, saccharify native celluloses and hemicelluloses, and ferment the resulting hexose and pentose monomers to ethanol, thus yielding appreciable amounts of ethanol from lignocellulosic feedstock. Therefore, the purpose of our program is to isolate, develop, and select Fusarium strains that hyperproduce ethanol from a given biomass material, as well as to optimize biohydrolysis and fermentation conditions to achieve maximum ethanol yields. This summary describes research conducted and results obtained during the period October 1981-March 1982 (i.e., the first half of FY 1982).

During early FY 1982, a few additional Fusarium isolates were obtained by using various media (previously described).[1] The new isolates were screened for their cellulolytic and fermenting abilities, and promising isolates were retested.

Research focused primarily on cellulose hydrolysis and on fermentation of glucose and xylose by selected Fusarium isolates. Preliminary screenings for cellulolytic ability were done by the cellulose-agar test-tube clearing assay, which identified certain isolates as potential cellulolyzers. Further testing of these isolates indicated that their cellulase production ranged mostly from 0.2 to 0.8 IU/mL after 14 days of processing.

In one study using a Fusarium isolate from strain ANL 22-760, cellulose (Solka Floc) was found to be the best inducer of FPase (filter-paper enzyme activity), CMCase (carboxymethyl cellulase), and xylanase, while glucose, lactose, and xylose almost completely inhibited cellulase production. The same isolate, when grown on 1% cellulose solution in the fermentor with an aeration rate of 0.05 v/v min^{-1}, produced 1.0 to 1.4 IU/mL cellulase in several fermentor runs. This isolate has served as parent in several UV irradiation mutation experiments.

Fusarium ANL 11182, an isolate derived from UV irradiation of ANL 8-72181, was used to optimize fermentor conditions for cellulase production. Tests with this isolate to determine carbon-source effect on enzyme yields indicated that a cellulase production of 2.5 IU/mL in 14 days was obtained when the medium contained 1% cellulose (Solka Floc), 0.02% Tween 80 (a polyoxyethylene surfactant), 1 g/L proteose peptone, 1 g/L NH_4NO_3, and 0.5 g/L yeast extract at a temperature of 28°C. The pH, which was left uncontrolled during this test, rose initially to 6.7, then dropped rapidly to 3.7; by the 14th day it had increased to 5.2. Increasing the nitrogen content of the medium stimulated cell growth but decreased cellulase production. Proteose peptone and yeast extract were not necessary for cellulase production, but were observed to increase the yield of cellulase when present in small amounts.

Examination of the effect of cellulose concentrations on cellulase yields showed that increasing the cellulose level to 2% caused a decrease of FPase activity over a 14-day period. Lowering the concentration to 0.5% resulted in initially higher rates of FPase activity, but overall activity was lower than with cellulose concentrations of 1%.

The ratio of CMCase to FPase remained nearly constant at about 10:1 during fermentations with varying concentrations of cellulose and different nitrogen sources, while the ratio of xylanase to FPase -- normally near 9:1 -- increased when supplemental xylan was added to the cellulose medium.

Several of the new Fusarium isolates and mutants, when screened for fermenting ability on glucose, produced ethanol at a conversion efficiency close to that achieved in yeast fermentations. Fermentations of 1% glucose with isolates from strain ANL 99A-780 repeatedly produced 4.3 mg/mL ethanol within 48 hours.

The fermentation of xylose to ethanol, another focus of our studies, is directed toward selection of Fusarium isolates and mutants that are

capable of efficient fermentation. In consecutive fermentations of 1% xylose, a few of the new isolates and mutants produced up to 4.2 mg/mL ethanol within 48 hours. The specially prepared Fusarium inoculum used for these fermentations consisted of centrifuged and pelletized spores and cells that were derived from a fermentation medium and recycled.

Fermentation of higher xylose concentrations was tested with some of the new isolates and mutants. Isolates from strain ANL 22-760 produced more than 8 mg/mL ethanol in 48 hr from 2% xylose solutions. Fermentations of 3% xylose solutions initially (in 48 hours) produced lower ethanol yields than those from 2% solutions, but by 68 hours the yield level had increased somewhat.

Addition of glucose to higher concentrations of xylose increased the yield of ethanol. For example, fermentation of a solution of 4.5% xylose plus 2% glucose produced more than 20 mg/mL ethanol in 72 hours, while higher concentrations of xylose reduced the ethanol yield.

2 INTRODUCTION

Recorded evidence indicates that selected Fusarium strains are able to partially free celluloses from their lignin mantle, saccharify native celluloses and hemicelluloses, and ferment the resulting hexose and pentose monomers to ethanol, thus yielding appreciable amounts of ethanol from lignocellulosic feedstock.[2-17] These strains are fungi that have a wide substrate range and are armed with a versatile series of enzymes. Like many other fungal strains, they release polysaccharide-splitting enzymes and assimilate the derived monomers for their energy and carbon requirements. Furthermore, F. strains seem to be equipped with an efficient enzymatic mechanism that enables them to glycolize and readily ferment monosaccharides.

Studies of carbon balance, enzymatic activity, and isotope distribution have indicated that the Embden-Meyerhoff-Parnas, or EMP, mechanism functions anaerobically in the F. strains to produce ethanol.[14,15] Fusaria are known to produce ethanol and carbon dioxide from hexoses in the same ratio as that achieved by yeast fermentations.[8,10,12] Birkinshaw and others[10] have found that the 23 species of Fusarium they investigated all give appreciable amount of ethanol. Many investigators have studied the fermenting ability of F. oxysporum f. sp. lini.[8,9,11,13,14,17] Pentose fermentation by F. strains has been studied by several authors who noted that an appreciable amount of ethanol was produced.[8,9,13,14,16,18] Letcher and Willaman[8] reported that not all isolates of a Fusarium species are equally efficient in yielding ethanol.

Based on this reported ability of Fusarium strains to degrade phytomass and yield ethanol via fermentation, the purpose of our program is to isolate, develop, and select Fusarium strains that hyperproduce ethanol from biomass, as well as to improve and optimize biohydrolysis and fermentation conditions to achieve maximum ethanol yields. This report describes research conducted and results obtained during the period October 1981-March 1982, i.e., the first half of FY 1982.

3 RESEARCH EFFORTS AND RESULTS

During the first half of FY 1982, research focused mostly on isolating and developing new Fusarium isolates and searching among them for potential cellulolyzers and glucose- and xylose-fermenters. These efforts and their results are described below.

3.1 ISOLATION AND DEVELOPMENT OF FUSARIA

Additional Fusarium isolates were obtained from sources such as soil, plant debris, roots, and partially delignified wood. The isolation medium was a modification of that used by Martin[19] and Papavizas[20] and contained 10.0 g dextrose, 5.0 g peptone, 0.5 g K_2HPO_4, 0.05 g rose bengal, 0.03 g streptomycin, 0.5 g pentachloronitrobenzene (PCNB), and 15.0 g agar per liter. Fusarium colonies were transferred to potato-dextrose-agar (PDA) for identification and then inoculated on PDA slants for maintenance and future use.

Ultraviolet irradiation was used to induce mutation in Fusarium cultures in an attempt to develop mutants superior to the parent isolates in saccharifying cellulose and fermenting sugars to ethanol. Samples of three- to five-day-old liquid cultures in 1% Solka floc were mildly centrifuged to remove residual cellulose and mycelium and to obtain a spore suspension. This spore suspension was again centrifuged, and the resulting pellet was washed and suspended. The spore count was adjusted to 4 million spores/mL with sterile distilled water. A 5-mL aliquot of the spore suspension was placed in a glass petri dish and exposed to ultraviolet radiation (254 nm). Irradiated spore suspensions were then diluted and spread on cellulose-agar plates. Plates were incubated at 30°C for three to five days and then at 50°C overnight and examined for clearing zones around developing colonies.

Most of the obtained isolates are of the Fusarium oxysporum type. All new isolates and mutants were tested for their ability to hydrolyze cellulose and ferment glucose and xylose.

3.2 POTENTIAL CELLULOLYTIC FUSARIA

New isolates and mutants were tested for cellulolytic activity in the cellulose-agar-test-tube-clearing assay of Rautela and Cowling.[21] All showed an ability to hydrolyze cellulose, and a few isolates and mutants were selected for further screening based on the rate and depth of clearing. The selected isolates were tested for extracellular production of cellulase in 200 mL of mineral salt media containing 1% Solka Floc + 0.1% proteose peptone (PP) + 0.1% yeast extract (YE) + 0.02% Tween 80 (a polyoxyethylene surfactant) in 500-mL flasks; agitation was used. Enzyme production in this test ranged as high as 0.8 IU/mL after 14 days of cellulose hydrolysis.

In one study using Fusarium strain ANL 22-760, it was found that cellulose (Solka Floc) was the best inducer of FPase (filter-paper enzyme activity), CMCase, and xylanase, while glucose, lactose, and xylose almost completely inhibited production of these enzymes (Table 1). The same strain,

Table 1 Effect of Carbon Source on Yields of Cellulase (FPase), Carboxymethyl Cellulase (CMCase), and Xylanase by Fusarium Strain ANL 22-760 (in IU/mL)

Carbon Source	FPase	CMCase	Xylanase
1% Cellulose (SW-40)	1.1	11.7	9.4
1% Carboxymethyl Cellulose	0.1	0.7	0.0
0.25% Cellobiose	0.1	0.0	0.4
0.25% Xylan	0.1	0.6	6.0
0.25% Xylan + 1% SW-40	1.0	6.7	14.8
1% Glucose	0.1	0.0	0.1
1% Lactose	0.0	0.0	0.0
1% Xylose	0.1	0.0	0.2

when grown in a 1% cellulose solution in the fermentor with an aeration rate of 0.05 v/v min^{-1}, produced 1.0 to 1.4 IU/mL cellulase in several fermentor runs.

Fusarium strain ANL 11182, an isolate derived from UV irradiation of ANL 8-72181, was used to optimize fermentor conditions for cellulase production. Tests with this isolate to determine carbon-source effect on enzyme yields indicated that a cellulase production of 2.5 IU/mL in 14 days was obtained (Figure 1) when the medium contained 1% cellulose (Solka Floc), 0.02% Tween 80, 1.0 g/L proteose peptone, 1.0 g/L NH_4NO_3, and 0.5 g/L yeast extract at a temperature of 28°C. The pH, which was left uncontrolled during this test, rose initially to 6.7, then dropped rapidly to 3.7; by the 14th day it had increased to 5.2. Increasing the nitrogen content of the medium stimulated cell-population growth but decreased cellulase yields. Proteose peptone and yeast extract were not necessary for cellulase production, but caused cellulase yields to increase when present in small quantities.

As Figure 1 shows, the ratio of CMCase to FPase remained nearly constant at about 10:1 during fermentations with varying concentrations of cellulose and different nitrogen sources, while the ratio of xylanase to FPase -- normally at about 9:1 -- increased when supplemental xylan was added to the cellulose medium.

Results of experimentation on the effect of cellulose concentrations on cellulase yields are illustrated in Figure 2. Increasing the cellulose level to 2% caused a decrease of FPase activity over a 14-day period. Lowering the concentration to 0.5% resulted in initially higher rates of FPase activity, but overall activity was lower than with cellulose concentrations of 1%.

The lag time between inoculation of the medium in the fermentor and detectable cellulase production is not shortened satisfactorily via different inoculation procedures, nutrient levels, nitrogen sources, cellulose concentrations, or aeration. Further studies, therefore, have been scheduled in an attempt to decrease this lag time.

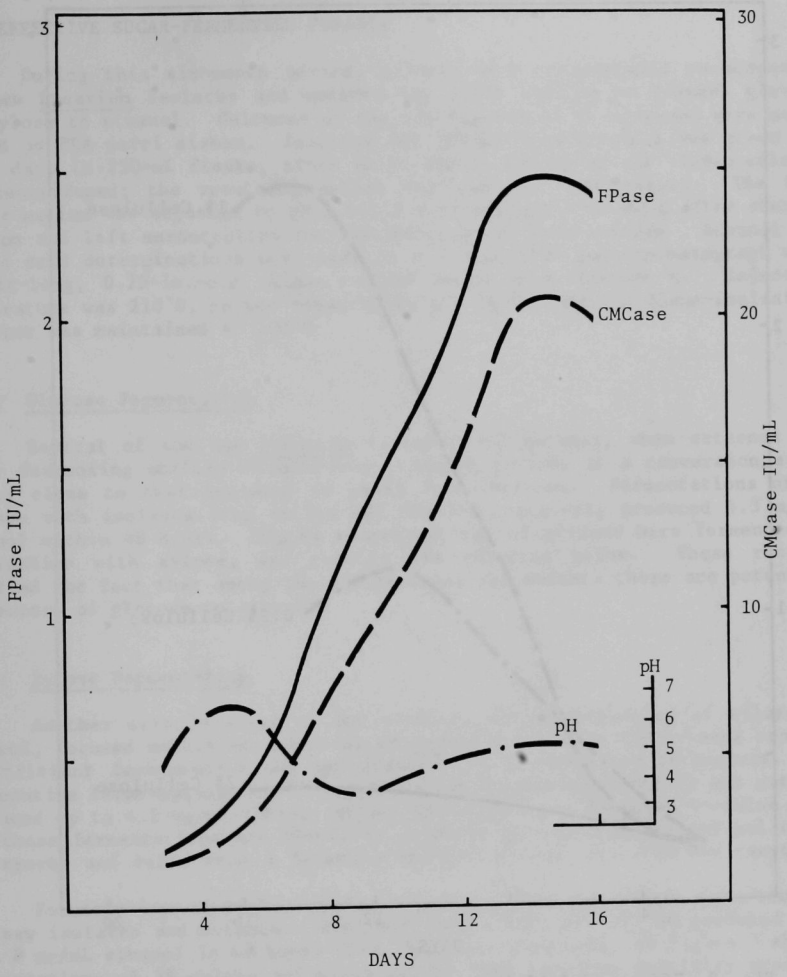


Fig. 1 Production of FPase and CMCase (in IU/mL) by Fusarium Strain ANL 11182 Grown on 1% Solka Floc in a 500-mL Fermentor, and Fluctuation of Medium pH vs. Time

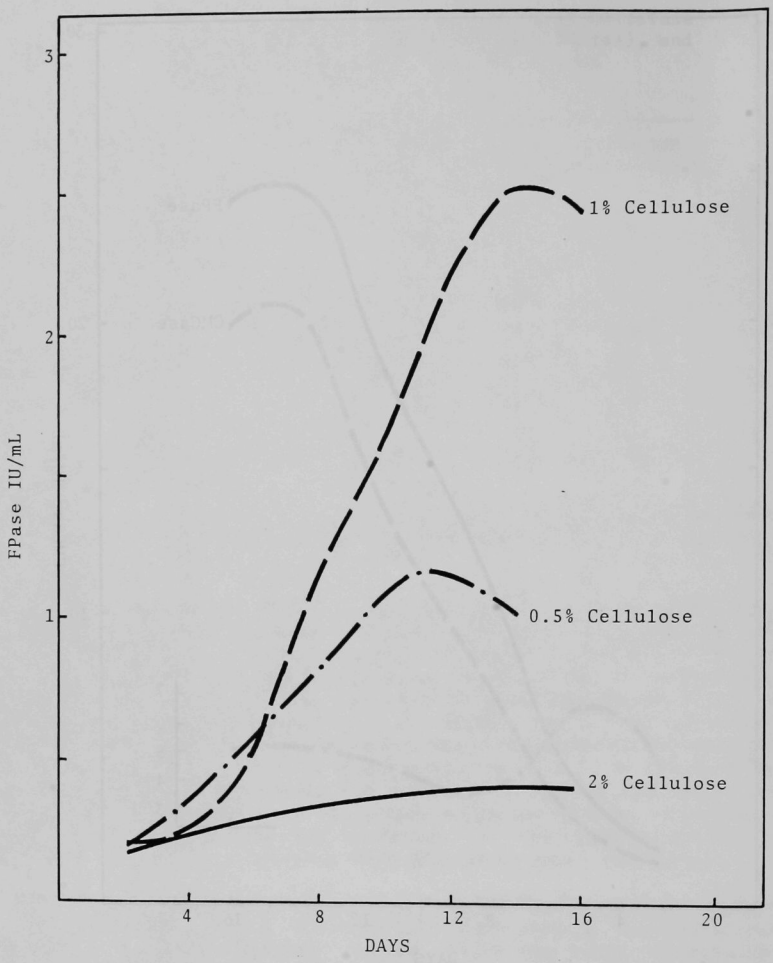


Fig. 2 Effect of Cellulose (Solka Floc) Concentration on FPase Production by Fusarium Strain ANL 11181 in a 500-mL Fermentor

3.3 EFFECTIVE SUGAR-FERMENTING FUSARIA

During this six-month period, efforts were concentrated on screening the new Fusarium isolates and mutants for their ability to ferment glucose and xylose to ethanol. Cultures of the new Fusaria to be screened were maintained on PDA petri dishes. Inoculum for 500-mL fermentations was grown for three days in 250-mL flasks, after which 100 to 150 mL of the liquid culture was centrifuged; the resulting pellet was used for inoculation. The fermentor medium was adjusted to pH 5.0-5.2 with hydrochloric acid after sterilization and left uncontrolled for the duration of fermentation. Ethanol and acetic acid determinations were made on a Varian 3700 gas chromatograph with a 6-ft-long, 0.25-in.-o.d. glass column packed with Porapak Q. Injection temperature was 210°C, column temperature was 163°C, and the flame-ionization detector was maintained at 220°C.

3.3.1 Glucose Fermentation

Several of the new Fusarium isolates and mutants, when screened for their fermenting ability on glucose, produced ethanol at a conversion efficiency close to that achieved in yeast fermentations. Fermentations of 1% glucose with isolates from strain ANL 99A-780 repeatedly produced 4.3 mg/mL ethanol within 48 hours. Higher concentrations of glucose were fermented in combination with xylose, and results are reported below. These results verified the fact that among the new isolates and mutants there are potential fermenters of glucose to ethanol.

3.3.2 Xylose Fermentation

Another main interest of our studies, the fermentation of xylose to ethanol, focused mainly on selection of Fusarium isolates and mutants capable of efficient fermentation and optimization of fermentation parameters. In consecutive fermentations of 1% xylose, a few of the new isolates and mutants produced up to 4.2 mg/mL ethanol within 48 hours. The Fusarium inoculum used for these fermentations was specially prepared by centrifuging and pelletizing spores and cells from a fermentation medium; the inoculum was recycled.

Fermentation of higher xylose concentrations was tested with some of the new isolates and mutants. Isolates from strain ANL 22-760 produced more than 8 mg/mL ethanol in 48 hours from 2% xylose solutions, as Figure 3 shows. Fermentations of 3% xylose solutions by the same isolates initially produced (within 48 hours) lower ethanol yields than those from 2% solutions, but by 68 hours the yield level was increased somewhat.

Addition of glucose to higher concentrations of xylose increased the yield of ethanol. For example, fermentation of a 4.5% xylose plus 2% glucose solution produced more than 20 mg/mL ethanol in 72 hours, while higher concentrations of xylose reduced the ethanol yield (Figure 3). These results agree with those obtained during previous experimentations in FY 1981.[1] Further studies will focus on use of higher concentrations of xylose, as well as different levels of glucose, to determine the optimum combination of xylose and glucose percentages in a solution that provides maximum ethanol yields when fermented by selected Fusarium strains.

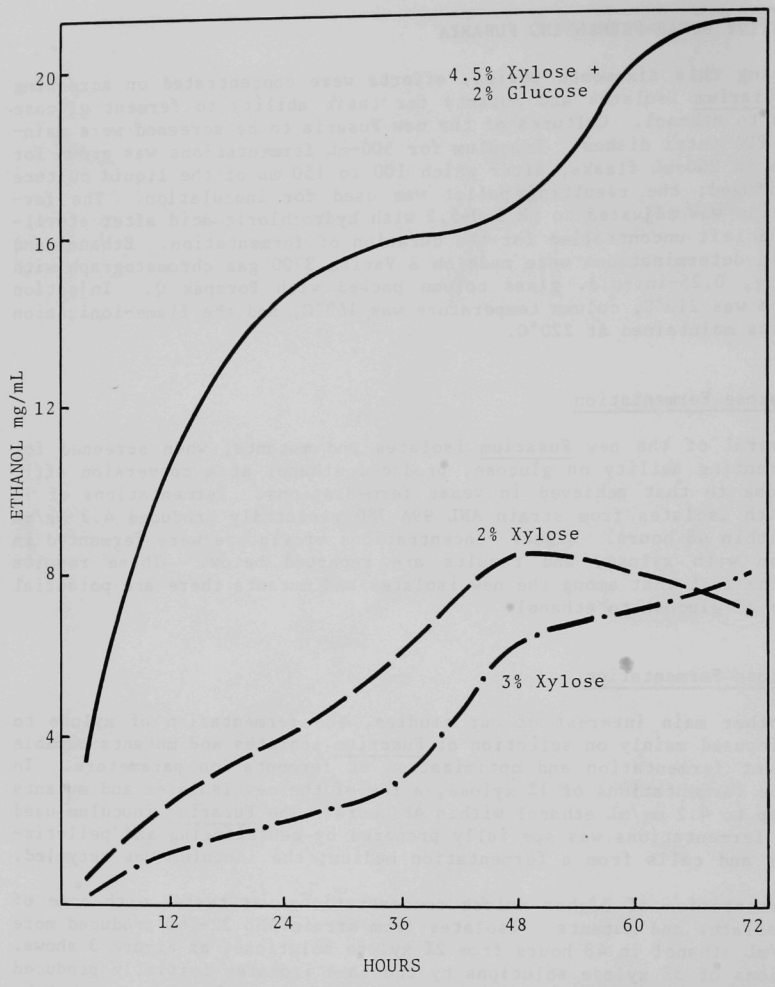


Fig. 3 Ethanol Yield (mg/mL) during Fermentation of 2% Xylose, 3% Xylose, and 4.5% Xylose plus 2% Glucose by an Isolate from Fusarium Strain ANL 22-760

4 RESEARCH PERSPECTIVES

As a continuation of this program, research will concentrate primarily on the following areas:

- Isolation of Fusarium strains that are capable of decomposing plant macromolecules for monosaccharide production.
- Selection of Fusarium strains that show superior lignocellulolytic ability.
- Genetic manipulation of Fusaria to develop superior lignocellulolytic and hexose- and pentose-fermenting strains.
- Determination of optimal parameters for better biomass saccharification and fermentation.
- Identification of end-product concentrations tolerable by Fusarium strains.
- Identification and quantification of enzymes involved and stimulation to increase and accelerate enzyme yield by Fusaria.
- Identification of synergistic and/or complementary actions of selected Fusarium strains and other microorganisms in saccharifying and fermenting biomass.

REFERENCES

1. Antonopoulos, A.A., and E.G. Wene, Ethanol Production via Fungal Decomposition and Fermentation of Biomass, Phase II (FY 1981) Annual Progress Report, Argonne National Laboratory Report ANL/EES/TM-162 (1981).

2. White, W.L., et al., Assay of Cellulolytic Activity of Molds Isolated from Fabrics and Related Items Exposed in the Tropics, Mycologia 40:34-84 (1948).

3. Husain, A., and A.E. Dimond, Production of Cellulase by Fusarium oxysporum f. lycopersici, Phytopathology 48:263 (1958).

4. Gascoigne, I.A., and M.M. Gascoigne, The Xylanases of Fusarium roseum, J. Gen. Microbiol. 21:242-248 (1960).

5. Sedha, R.K., H.K. Tewari, and D.S. Chalal, Synthesis of Cellulases in Aspergillus terreus and Fusarium sp., Indian J. Microbiol. 18:93-96 (1978).

6. Wood, T.M., and S.I. McCrae, The Cellulase of Trichoderma koningii, Purification and Properties of Some Endoglucanase Components with Special Reference to Their Action on Cellulose when Acting Alone and in Synergism with Cellobiohydrolase, Biochem. J. 171:61-72 (1978).

7. Svabova, M., et al., Enzyme Apparatus of the Genus Fusarium, Folia Microbiol. 25:233-237 (1980).

8. Letcher, H., and J.J. Willaman, Biochemistry of Plant Diseases, VIII, Alcoholic Fermentation of Fusarium lini, Phytopathology 16:941-949 (1926).

9. White, M.G., and J.J. Willaman, Biochemistry of Plant Diseases, X. Fermentation of Pentoses by Fusarium lini, Biochem. J. 22:583-591 (1928).

10. Birkinshaw, J.H., et al., Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products Formed from Glucose by Species of Fusarium, in Studies in the Biochemistry of Microorganisms, H. Raistrick et al., Philosophical Transactions (London) 222(-467B):93-98 (1931).

11. Anderson, A.K., E.L. Everitt, and P.D. Adams, The Carbon Metabolism of Fusarium oxysporum on Glucose, J. Agric. Research 46:473-482 (1933).

12. Nord, F.F., and S. Weiss, Yeast and Mold Fermentations, in The Enzymes, J.B. Summer and K. Myrback, eds., Vol. 2, Part 1, pp. 684-790, Academic Press, New York (1951).

13. Gibbs, M., et al., Fermentation of D-Xylose-1-C^{14} by Fusarium lini Bolley, Arch. Biochem. and Biophys. 50:237-242 (1954).

14. Cochrane, V.W., The Anaerobic Dissimilation of Glucose by Fusarium lini, Mycologia 48:1-12 (1956).

15. Heath, E.C., D. Nasser, and H. Koffler, Biochemistry of Filamentous Fungi, III, Alternative Routes for the Breakdown of Glucose by Fusarium lini, Arch. Biochem. and Biophysics 64:80-87 (1956).

16. Batter, T.R., and C.R. Wilke, Study of the Fermentation of Xylose to Ethanol by Fusarium oxysporum, Lawrence Berkeley Laboratory-Univ. of California at Berkeley report (1977).

17. Rosenberg, S.L., et al., Hemicellulose Utilization for Ethanol Production, Lawrence Berkeley Laboratory paper (1980).

18. Wilke, C.R., Process Development Studies on the Bioconversion of Cellulose and Production of Ethanol, Lawrence Berkeley Laboratory-Univ. of California at Berkeley report (1978).

19. Martin, J.P., Use of Acid, Rose Bengal, and Streptomycin in the Plate Method for Estimation of Soil Fungi, Soil Sci. 69:215-232 (1950).

20. Papavizas, G.C., New Medium for the Isolation of Thielaviopsis bassicola on Dilution Plates from Soil and Rhizosphere, Phytopathology 54:1475-1481 (1964).

21. Rautela, G.S., and E.G. Cowling, Simple Cultural Test for Relative Cellulolytic Activity of Fungi, Appl. Microbiol. 14:892-898 (1966).